Specific Inner Retinal Layer Cell Damage in an Autoimmune Glaucoma Model Is Induced by GDNF With or Without HSP27

Christina Casola,[1] Sabrina Reinehr,[1] Sandra Kuehn,[1] Gesa Stute,[1] Bernhard M. Spiess,[2] H. Burkhard Dick,[1] and Stephanie C. Joachim[1]

[1]Experimental Eye Research Institute, University Eye Hospital, Ruhr-University Bochum, Bochum, Germany
[2]Equine Department, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

Correspondence: Stephanie C. Joachim, Experimental Eye Research Institute, University Eye Hospital, Ruhr-University Bochum, In der Schornau 23-25, 44892 Bochum, Germany; stephanie.joachim@rub.de.

Submitted: December 21, 2015
Accepted: May 19, 2016

Citation: Casola C, Reinehr S, Kuehn S, et al. Specific inner retinal layer cell damage in an autoimmune glaucoma model is induced by GDNF with or without HSP27. *Invest Ophthalmol Vis Sci*. 2016;57:3626–3639. DOI:10.1167/iovs.15-18999R2

Purpose. Previously, immunization of rats with ocular antigens induced retinal ganglion cell (RGC) degeneration. We investigated the effect of immunization with glial cell line-derived neurotrophic factor (GDNF) or GDNF in combination with heat shock protein 27 (GDNF+HSP) on RGCs and other retinal cells.

Methods. Rats were immunized with GDNF or GDNF+HSP. After 4 weeks, retinas were stained with Brn-3a and NeuN to quantify RGCs. GFAP and vimentin staining were used to investigate macroglia. Microglia were marked with Iba1 and ED1. Amacrine cells were labeled with parvalbumin and ChAT. Photoreceptors were evaluated with rhodopsin and opsin staining and bipolar cells with PKCα and recoverin. For these cell types, Western blotting was also performed.

Results. Retinas of immunized animals showed a significant loss of Brn-3a$^+$ and NeuN$^+$ RGCs. No significant changes could be observed in regard to macroglia. An increase in Iba1$^+$ microglia was detected in both groups, but little change in regard to activated microglia. A loss of cholinergic amacrine cells was seen in the GDNF+HSP group by immunohistochemistry and in both groups via Western blot analysis. AII amacrine cells, bipolar cells, and photoreceptors were not affected.

Conclusions. Immunizations led to loss of RGCs and cholinergic amacrine cells and a strong increase in microglial cells. Our data suggest that RGC loss is the consequence of immunization with GDNF. Astrocyte activity and its neuroprotective effects seem to be inhibited by GDNF immunization. We presume more complex interactions between GDNF and HSP27 because no additive effects could be observed.

Keywords: amacrine cells, glaucoma, glial cell line-derived neurotrophic factor, heat shock protein 27, retinal ganglion cells

There are many different possibilities for classification of glaucoma, but it is always defined by a progressive loss of retinal ganglion cells (RGCs) and their axons and is therefore categorized as a neurodegenerative disease.[1] Glaucoma with normal tension constitutes approximately 30% of glaucoma cases worldwide.[2] As opposed to high-pressure glaucoma, the normotensive type leads to irreversible blindness without existence of elevated intraocular pressure (IOP). Glaucoma pathogenesis is still poorly understood, which is why no curative therapy could be found until now.

Currently, there is growing evidence for an involvement of the immune system in the disease of glaucoma.[3] Increased levels of autoantibodies against heat shock protein 27 (HSP27) were detected in glaucoma patients who did not have an elevated IOP.[4,5] Later, a high variance of autoantibodies against optic nerve antigens were described in glaucoma patients with normal tension.[6,7] These changes in antibody patterns of up- and downregulation could be observed not only systemically but also in ocular fluids.[8] HSP27 was identified as one of the potentially important antigens in glaucoma, especially normal tension glaucoma.[9,10] In cell cultures, it was demonstrated that antibodies applied directly against small heat shock proteins on retinal cells resulted in apoptosis.[5] Cytotoxic effects occurred at concentrations of HSP antibodies similar to those found in the sera of many glaucoma patients, which suggests a direct cause rather than an epiphenomenon.[9] However, these findings could not yet confirm that RGC loss in glaucoma is a direct result of aberrant immunity. Therefore, in vivo experiments were necessary and an autoimmune glaucoma model was developed. This animal model was established on the basis of immunization models for the evaluation of other diseases (e.g., the experimental autoimmune encephalomyelitis model for multiple sclerosis).[11,12] In those models, animals are also immunized systemically in order to evaluate the response of the central nervous system.

Previously, rats were immunized with an optic nerve antigen homogenate, which is a mixture of optic nerve antigens. This immunization led to a loss of RGCs. There was also a correlation between duration after immunization and the existence of autoreactive antibodies.[13] Antibody deposits in the retina after immunization provided further evidence for an involvement of the immune system in the disease of

Investigative Ophthalmology & Visual Science

glaucoma.[14] The exact pathway of cell death is still unknown in this model, but many studies indicate that autoreactive antibodies could play a role in the pathogenesis. The goal of our present and future studies is to find out which particular antigens are important in glaucoma disease progress.

In previous studies, immunization with HSP27, a purified antigen, led to loss of RGCs.[15,16] In addition to autoantibodies to HSP27, elevated antibodies against the glial cell line-derived neurotrophic factor (GDNF) were found in glaucoma patients with normal tension. It is still unclear if the antibodies found in glaucoma patients are the cause or the consequence of the disease. In a pilot study, immunization with GDNF led to a significant loss of RGCs and was therefore chosen for the study (Joachim SC, unpublished data, 2014).

The purposes of the present study were to determine if immunization with GDNF alone or GDNF in combination with HSP27 (GDNF+HSP) would lead to a loss of RGCs and to investigate effects on other retinal cells. We were able to reveal a significant loss of RGCs, an increase in microglial cells and a loss of cholinergic amacrine cells in both immunization groups.

Materials and Methods

Animals

Male Lewis rats between 7 and 8 weeks of age (Charles River, Sulzfeld, Germany) were kept in a constant temperature and received food and water ad libitum. Illumination was adjusted to a 12/12-hour dark/light cycle. Daily examinations of general condition and welfare were performed, including observation of ophthalmic disorders. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the animal care committee of North Rhine-Westphalia (Germany; AZ 87-51.04.2010.A382 and AZ 23177-07; G 09-1-044).

Immunization

Rats were randomly divided in 2 immunization groups and 1 control group (Co; n = 4–5 animals/group). In previous studies, the dose of 100 μg of purified HSP27 caused retinal ganglion cell damage.[15,17] We wanted to evaluate additive effects and therefore chose the following concentrations.

Each animal in the GDNF group received 200 μg of GDNF (Biotrend Chemicals, LLC, Destin, FL, USA). Animals in the GDNF+HSP group were injected with 100 μg of GDNF plus 100 μg of HSP27 (AtGen, Yatap-dong, South Korea), whereas animals in the control group received 200 μL of a 0.9% sodium chloride solution.[15,17] Simultaneously, 3 μg of pertussis toxin and 200 μL of Freund's adjuvant were administered to each animal. Solutions were given by intraperitoneal injection.

Animals were checked daily for 1 week after immunization, then twice a week. According to a checklist, attention was given to coordination, possible swelling of the injection site, behavior, respiration, healthy mouth, stable or increasing weight, normal feces, and possible hemorrhage. Eyes were examined for blepharospasm, ocular discharge, redness, chemosis, and opacities of the cornea or lens. Parameters were evaluated using a scoring system, and all rats achieved a condition score of zero throughout the whole study, which meant that no abnormalities were found.

Measurement of Intraocular Pressure

Intraocular pressure was measured after instillation of a topical anesthetic (Novesine 0.4%; Omni Vision GmbH, Puchheim, Germany), using a TonoPen XL (Medtronic, Baseweiler, Germany).[18] Measurements were performed before, then at 2, and at 4 weeks after immunization, always at the same time of day by the same examiner.

Funduscopy

To manage funduscopy, the rats had to be generally anesthetized. A mydriatic (Mydriatikum Stulln, Pharma Stulln, Germany) was applied topically. A glass slide with contact medium (Methocel 2%; Omni Vision) was carefully pressed onto the locally anesthetized cornea. Then fundi were examined through a binocular surgical microscope (Carl Zeiss, Jena, Germany) in search of signs of damage at 2 and 4 weeks after immunization.

Tissue Preparation for Retinal Cross-Sections and Histology

Four weeks after immunization, rat eyes were enucleated and fixed in 4% paraformaldehyde. Thereafter, one eye of each animal (n = 4–5/group) underwent a sucrose treatment, was embedded in compound (Tissue-Tek; Fisher Scientific, Schwerte, Germany), and stored at a temperature of –80°C. Cross-sections 10 μm thick were made with a cryostat (Fisher Scientific) for further staining. For an overview, cross-sections (n = 5 eyes/group; 4 cross-sections/eye) were evaluated by light microscopy (Axio Imager M1; Zeiss) after staining with hematoxylin and eosin (H&E) and cresyl violet (Nissl) by using standard protocols.[19] Retinas of the other eye were prepared for Western blotting.

Histology of Retinal Cell Types

In order to identify the different retinal cell types, specific antibodies were used for immunofluorescence staining (n = 4–5/group; 6 sections/animal) (Table). Briefly, sections of the retina were blocked with a solution containing donkey and/or goat serum and 0.1% to 0.2% Triton-X in PBS. Sections were incubated with primary antibodies at room temperature overnight. The next day, incubation with corresponding secondary antibodies was performed for 1 hour. Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI; Dianova, Hamburg, Germany; or Serva Electrophoresis, Heidelberg, Germany) was included to facilitate orientation on the slides. Negative controls were performed for each stain by using secondary antibodies only.

Histologic Examination

The photographs were taken using a fluorescence microscope (Axio Imager model M1; Zeiss). Two photos of the peripheral and two of the central part of each section were captured for each group. Images were transferred to Paint Shop Pro version 13 software (Corel Corp., CA, USA), and equal excerpts were cut out. RGCs, microglia, amacrine cells, bipolar cells, and cones were counted using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Measurements and analyses of GFAP-, vimentin-, and rhodopsin-labeled areas were evaluated using ImageJ software, as described previously.[19] Briefly, images were transformed into grayscale. A fixed background with a rolling ball radius of 50.0 pixels was subtracted from GFAP and vimentin images. The percentage (%) of the labeled area was then measured between defined thresholds (GFAP lower threshold was 4.18; upper threshold was 66.98; vimentin lower threshold was 9.20; upper threshold was 67.42; and

TABLE. Antibodies Used for Histology and Western Blot

Primary Antibody				Secondary Antibody			
Antibody	Company	Application	Dilution	Antibody	Company	Application	Dilution
β-III tubulin	R&D Systems	Western blot	1:1000	Donkey anti-mouse Alexa Fluor 680	Invitrogen	Western blot	1:5000
Brn-3a	Santa Cruz	Histology	1:100	Donkey anti-goat Alexa Fluor 488	Dianova	Histology	1:400
ChAT	Millipore	Histology	1:100	Goat anti-mouse Alexa Fluor 488	Invitrogen	Histology	1:500
		Western blot	1:500	Donkey anti-mouse Alexa Fluor 680	Invitrogen	Western blot	1:5000
ED1	Millipore	Histology	1:250	Goat anti-mouse Alexa Fluor 488	Invitrogen	Histology	1:500
GFAP-A488	Millipore	Histology	1:1200				
GFAP	Millipore	Western blot	1:3000	Donkey anti-chicken IRDye 680RD	LI-COR	Western blot	1:20000
Iba1	Wako	Histology	1:400	Goat anti-rabbit Cy3	Linaris	Histology	1:500
NeuN	Millipore	Histology	1:500	Donkey anti-chicken Cy3	Millipore	Histology	1:400
Opsin	Millipore	Histology	1:200	Donkey anti-rabbit Alexa Fluor 555	Invitrogen	Histology	1:500
Parvalbumin	Swant	Histology	1:100	Donkey anti-goat Alexa Fluor 488	Dianova	Histology	1:400
PKCα	Santa Cruz Biotechnology	Histology	1:500	Goat anti-mouse Alexa Fluor 488	Invitrogen	Histology	1:500
Recoverin	Millipore	Histology	1:1000	Donkey anti-rabbit Alexa Fluor 555	Invitrogen	Histology	1:400
		Western blot	1:2000	Donkey anti-rabbit Alexa Fluor 680	Invitrogen	Western blot	1:5000
Rhodopsin	Abcam	Histology	1:400	Goat anti-mouse Alexa Fluor 488	Invitrogen	Histology	1:500
		Western blot	1:1000	Donkey anti-mouse Alexa Fluor 680	Invitrogen	Western blot	1:5000
TSPO	Abcam	Western blot	1:100	Donkey anti-rabbit Alexa Fluor 680	Invitrogen	Western blot	1:5000
Vimentin	Sigma-Aldrich	Histology	1:500	Goat anti-mouse Alexa Fluor 555	Invitrogen	Histology	1:400
		Western blot	1:500	Goat anti-mouse IgM IRDye 680RD	LI-COR	Western blot	1:20000
β-actin	Sigma-Aldrich	Western blot	1:6000	Donkey anti-mouse DL800	LI-COR	Western blot	1:20000
β-actin	Cell Signaling	Western blot	1:6000	Donkey anti-rabbit DL800	Thermo Fisher Scientifc	Western blot	1:5000

rhodopsin lower threshold was 11.02; upper threshold was 54.51).

Western Blot Analysis

Retinas were prepared for Western blot analysis (n = 3-4/group) as previously described.[20] To isolate proteins, the frozen retinas were mechanically homogenized in 150 μL of lysis buffer (Radioimmunoprecipitation assay buffer; Cell Signaling Technology, Danvers, MA, USA) and a protease inhibitor (Sigma-Aldrich Corp., St. Louis, MO, USA). Ultrasound was then used for an accurate homogenization. Samples were placed on ice for 50 minutes, followed by centrifugation at 4°C at 13,200 rpm for 30 minutes to reduce the last solid particles. The protein concentration was measured using a commercial bicinchoninic acid assay (BCA protein assay kit; Pierce Technology, Holmdel, NJ, USA). We applied 20 μg per lane to a 12% bis-Tris gel (NuPAGE; Invitrogen, Carlsbad, CA, USA) for electrophoresis and blotted afterward, using a transfer buffer (NuPAGE) for 60 minutes at 200 V. Nitrocellulose membranes were blocked in a solution of 5% milk powder/PBS/0.05% Tween-20. Primary antibodies were applied and incubated overnight, followed by secondary antibodies for 60 minutes (Table). An Odyssey infrared imager system 2.1 (LI-COR Biosciences, Lincoln, NE, USA) recorded and analyzed the stained protein bands. Two measurements of each animal were used for statistical analysis.

Statistics

Cell counts, evaluated area fractions, and Western blot data were compared by ANOVA followed by Dunnett post hoc test. IOP was analyzed using ANOVA, followed by Tukey post hoc test. Data are mean ± SEM. Statistical analysis was performed by Statistica version 12 software (StatSoft, Tulsa, OK, USA).

RESULTS

In Vivo Parameters

Animals were in a good condition during the whole study. Daily examination of the rats showed no abnormal findings. Special attention was given to coordination, behavior, movement, orientation, and signs of paralysis. Eyes showed no signs of inflammation or discomfort.

Time Course of Intraocular Pressure and Funduscopy

At baseline, no differences in IOP could be noted in all immunization groups compared to control (Co: 11.81 ± 0.45 mm Hg; GDNF: 11.81 ± 0.34 mm Hg; GDNF+HSP: 10.93 ± 0.15 mm Hg; $P > 0.05$). Two weeks after immunization, IOP stayed in a normal range in all groups (Co: 12.02 ± 0.25 mm Hg; GDNF: 11.76 ± 0.3 mm Hg; GDNF+HSP: 11.2 ± 0.18 mm Hg; $P > 0.05$). Also, after 4 weeks, we could not find any IOP elevation by comparison between the immunized groups and control (Co: 11.45 ± 0.23 mm Hg; GDNF: 11.66 ± 0.15 mm Hg; GDNF+HSP: 11.39 ± 0.24 mm Hg; $P > 0.05$) (Fig. 1B).

Funduscopy revealed no abnormalities of the optic nerve head or of retinal vasculature in the control group or in the immunization groups after 2 and 4 weeks (Fig. 1A).

Retinal Histology

Retinal cross-sections were stained with H&E (Fig. 2A) and cresyl violet (Fig. 2B) to get an overview of changes in structure. Retinal layers were always well defined, and no infiltrates or signs of inflammation could be seen. In comparison, the control retinas and those of the immunization groups showed no difference in histology.

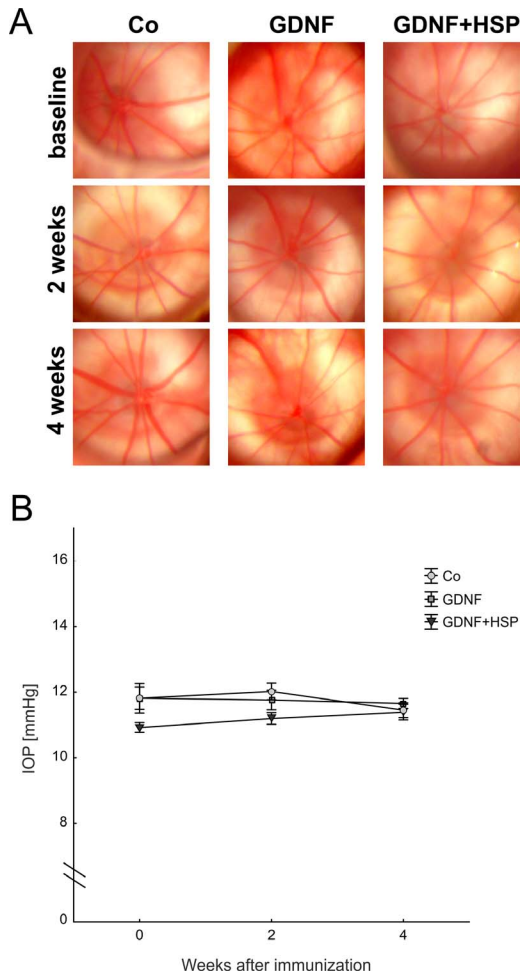

FIGURE 1. (**A**) Funduscopy photos of retinas of control and immunized animals at different points in time. No pathologic changes or signs of inflammation could be seen. (**B**) Mean IOP levels at baseline and two as well as four weeks after immunization. IOP remained in the normal range throughout the whole study and showed no difference between the immunization groups compared to control ($P > 0.05$). Values represent mean ± SEM; $n = 5$/group.

Retinal Ganglion Cell Loss

To improve reliability of cell counts, two different markers for RGC evaluation were used. Brn-3a is a specific marker for RGCs, whereas NeuN stains all neuronal cells. Most of the labeled cells displayed colocalization of the stains (Fig. 3A). The small amount of solely NeuN-stained cells were likely to be dislocated amacrine cells.[21] Retinas of immunized animals showed a significant loss of Brn-3a-stained RGCs (Co: 29.91 ± 2.14 cells/mm; GDNF: 20.01 ± 2.77 cells/mm; $P = 0.01$; GDNF+HSP27: 21.35 ± 0.21 cells/mm; $P = 0.04$) (Fig. 3C). These results were confirmed by Western blot analysis using an anti-β-III tubulin antibody (Fig. 3B), another marker for RGCs.[22] The β-III tubulin levels of the GDNF group (Co: 50.17 ± 10.8 units; GDNF: 11.95 ± 5.1 units; $P = 0.01$) and of the GDNF+HSP group (12.4 ± 2.8 units; $P = 0.02$) (Fig. 3D) were decreased compared to those of controls.

Also, the number of NeuN-labeled cells was significantly reduced in both groups compared to controls (Co: 30.08 ± 1.4 cells/mm; GDNF: 22.5 ± 2.69 cells/mm; $P = 0.03$; GDNF+HSP27: 22.29 ± 0.76 cells/mm; $P = 0.03$) (Fig. 3E).

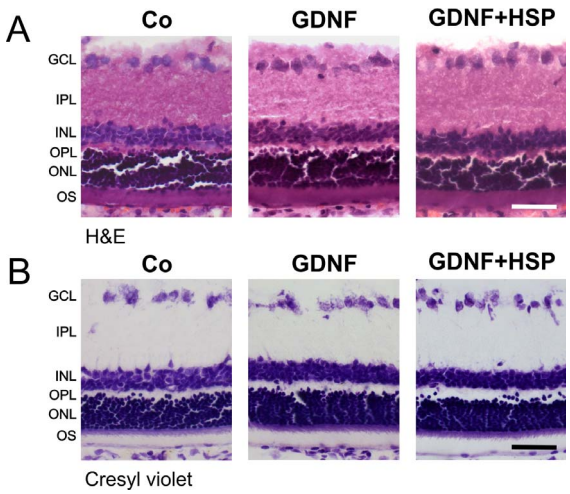

FIGURE 2. (**A**) Staining with H&E visualizes the retinal layers. Retinas of the immunization groups showed no inflammation or damage of retinal layers; morphology was comparable to that of controls. (**B**) Cresyl violet staining reveals normal organization of retinal layers in all groups. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segment. *Scale bar*: 40 μm.

No Macroglial Alteration

Gliosis seems to be an epiphenomenon of neuronal damage. Therefore, astrocytes and Müller cells were analyzed using GFAP or vimentin area analysis (Fig. 4A). In the GDNF group (10.35 ± 1.53 area [%]/section), the GFAP$^+$ area was slightly increased compared to that in the control group (6.56 ± 0.92 area [%]/section; $P = 0.1$) (Fig. 4C). In the GDNF+HSP group (7.19 ± 1.42 area [%]/section), no increase in the GFAP stained area was noted ($P = 0.9$) (Fig. 4C).

GFAP levels in retinas were also investigated by using Western blot analysis (Fig. 4B). The GFAP protein level, measured at 55 kDa, revealed no changes in the GDNF group (Co: 14.33 ± 2.48 units; GDNF: 15.75 ± 2.75 units; $P = 0.9$) (Fig. 4D). Also, no alterations were noted in the GDNF+HSP group (17.89 ± 3.32 units; $P = 0.6$) (Fig. 4D).

The vimentin$^+$ areas showed no alterations in the GDNF group (Co: 12.93 ± 1.91 area [%]/section; GDNF: 15.12 ± 1.59 area [%]/section; $P = 0.5$) or in the GDNF+HSP group (14.21 ± 2.20 area [%]/section; $P = 0.8$) (Fig. 4E). For vimentin with a molecular weight of 58 kDa, no increase in the protein level in the GDNF group (Co: 8.27 ± 2.9 units; GDNF: 8.19 ± 1.74 units; $P = 0.99$) or in the GDNF+HSP group was observed (4.58 ± 0.11 units; $P = 0.5$) by Western blot analysis (Fig. 4F).

Increased Numbers of Resting Microglia Cells

The whole population of microglia was labeled with Iba1. To identify activated microglia, the sections were also stained with ED1 (Fig. 5A). Additionally, activated microglia were quantified using translocator protein (TSPO) in Western blot analysis.

Figure 5A shows cell bodies and dendrites of microglia stained (red) with Iba1. Colocalization with ED1 (green), as seen in the merged picture, identifies the cell as active microglia. Significantly more Iba1$^+$ cells were revealed in the GDNF group (Co: 5.81 ± 0.82 cells/mm; GDNF: 21.16 ± 2.42 cells/mm; $P < 0.001$). Also, a significantly higher number of Iba1$^+$ cells was noted in the GDNF+HSP group (18.31 ± 0.78 cells/mm; $P < 0.001$) (Fig. 5E). Evaluation of the activation marker ED1 in combination with Iba1 showed no significant differences

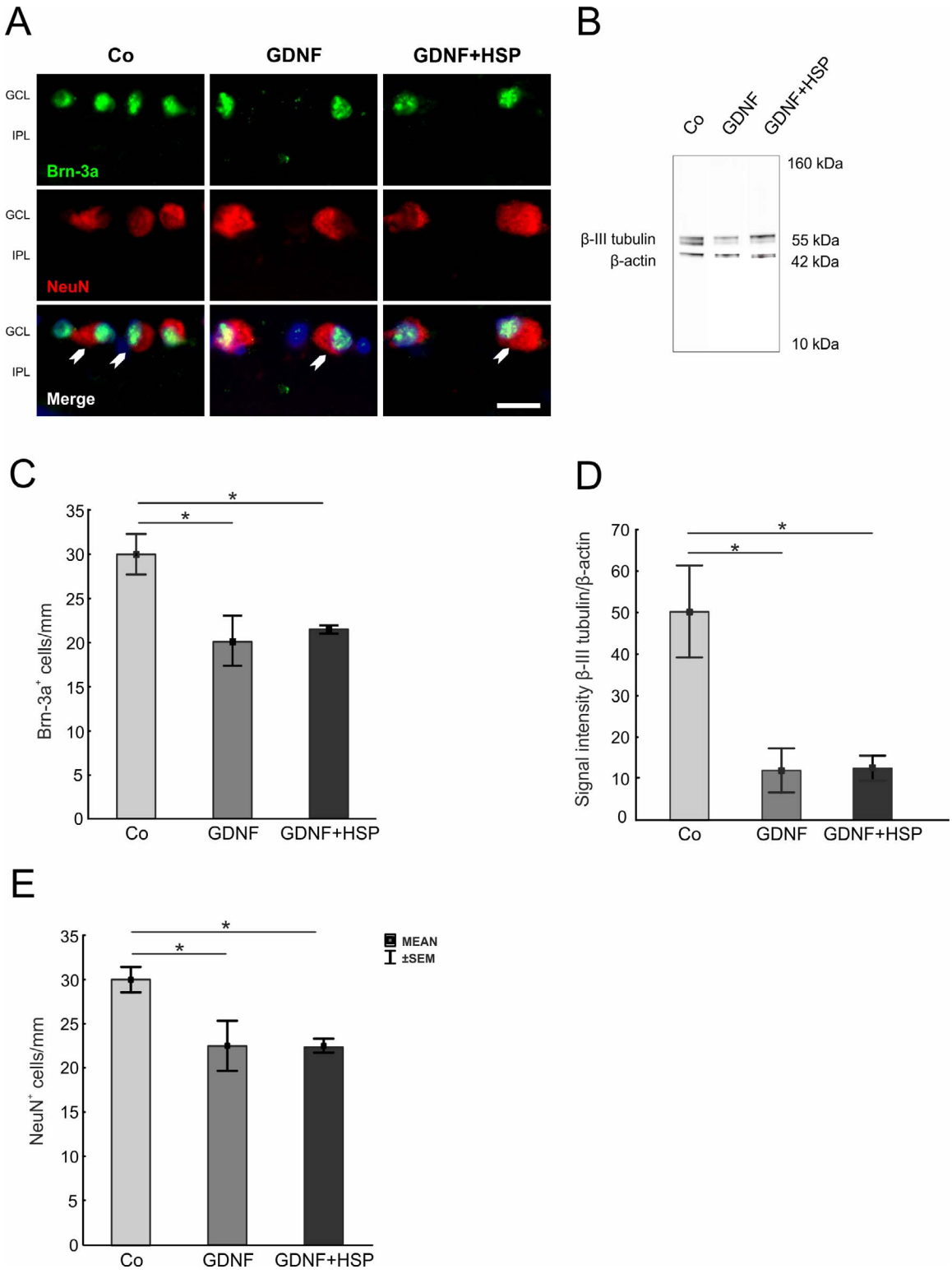

Figure 3. (**A**) Exemplary cross-sections of retina stained with Brn-3a (*green*), NeuN (*red*), and DAPI (*blue*) to quantify RGCs. *Arrows* indicate colocalization of Brn-3a- and NeuN-stained cells. (**B**) Western blot bands for β-III tubulin and β-actin. A less intense β-III tubulin staining for immunization groups can be observed. (**C**) Retinas of both of the immunization groups showed a significant loss of RGCs when labeled with Brn-3a (GDNF: $P = 0.01$; GDNF+HSP: $P = 0.04$). (**D**) Western blot analysis revealed a significant decrease in β-III tubulin staining in both of the immunization groups (GDNF: $P = 0.01$; GDNF+HSP: $P = 0.02$). (**E**) A significant loss of RGCs was also detected using NeuN staining (GDNF: $P = 0.03$; GDNF+HSP: $P = 0.03$). Values are mean ± SEM. GCL, ganglion cell layer; IPL, inner plexiform layer; IF, $n = 4$ to 5/group; Western blot (WB), $n = 3$ to 4/group. *Scale bar*: 20 μm.

Investigative Ophthalmology & Visual Science

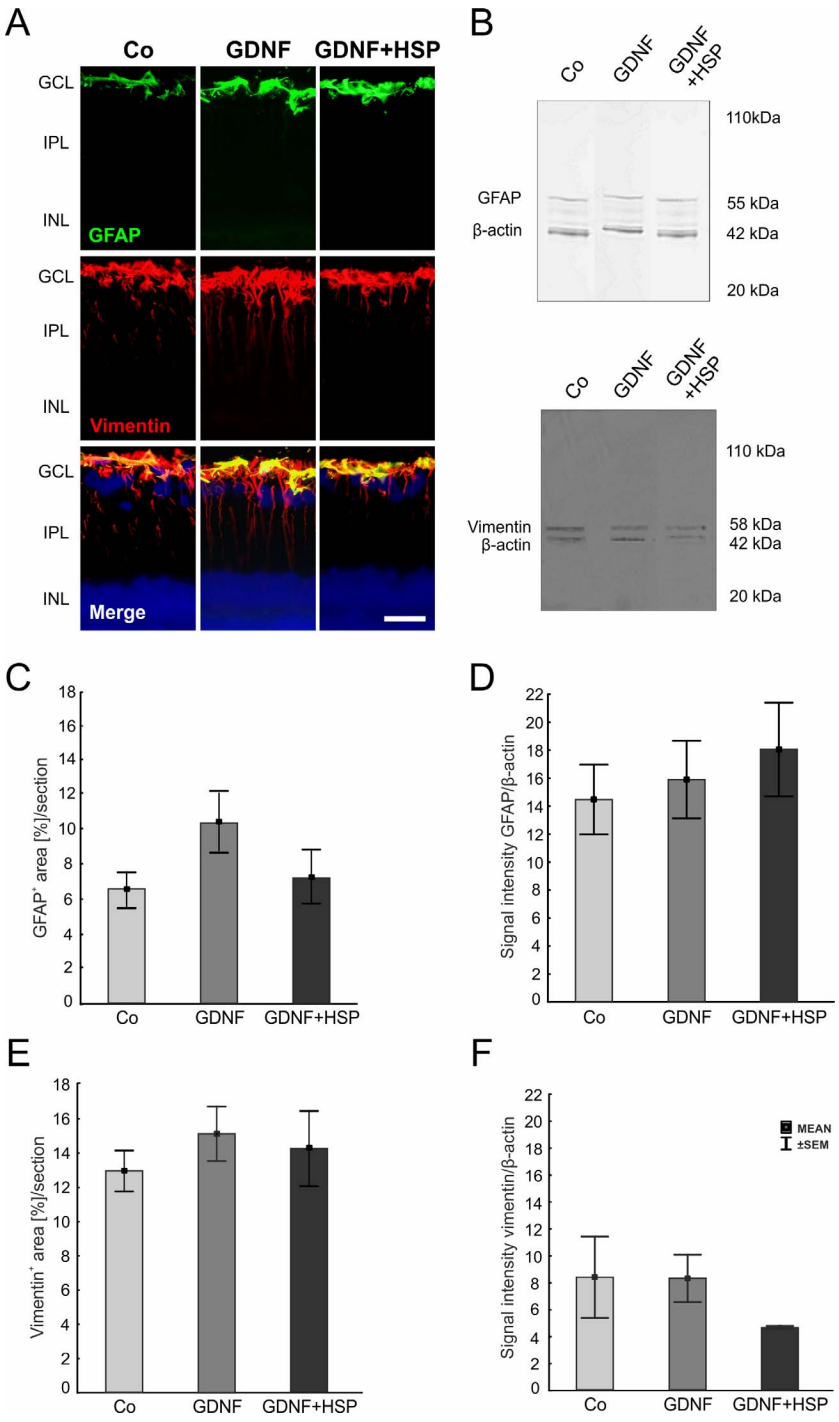

Figure 4. (**A**) Retinal sections were stained with GFAP (*green*), vimentin (*red*), and DAPI (*blue*) to detect possible alterations in macroglia. GFAP is sensitive mainly to astrocytes, whereas vimentin is expressed mostly in Müller cells. (**B**) Representative Western blot images of GFAP, vimentin, and corresponding β-actin bands from retinas of control and immunized animals. (**C**) No significant changes could be observed in regard to GFAP area in the GDNF ($P = 0.1$) or GDNF+HSP group ($P = 0.9$). (**D**) Western blot analysis results showed no differences between GFAP staining in the GDNF group ($P = 0.9$) and that in the GDNF+HSP group ($P = 0.6$) compared to staining in controls. (**E**) Vimentin staining revealed no alterations in either of the immunization groups ($P > 0.05$). (**F**) No differences were observed between Western blot analysis of vimentin in the GDNF group ($P = 0.99$) and that in the GDNF+HSP group ($P = 0.5$). Values are mean ± SEM. GCL, ganglion cell layer; IPL, inner plexiform layer; IF, $n = 4$ to 5/group; WB. $n = 3$ to 4/group. *Scale bar*: 20 μm.

between GDNF animals (10.00 ± 4.05 cells/mm) and controls (1.68 ± 0.26 cells/mm) (Fig. 5C). No changes were observed in the GDNF+HSP group (5.16 ± 1.07 cells/mm; $P = 0.6$).

Western blots were stained with TSPO, which is upregulated in microglia during neuroinflammation.[23–26] Western blot bands were measured at a molecular weight of 18 kDa (Fig. 5B). In accordance with ED1 results, no differences could be detected in the GDNF group (0.83 ± 0.28 units; $P = 0.71$) and in the GDNF+HSP group (0.87 ± 0.08 units; $P = 0.67$) compared to the control (0.65 ± 0.11 units) (Fig. 5D).

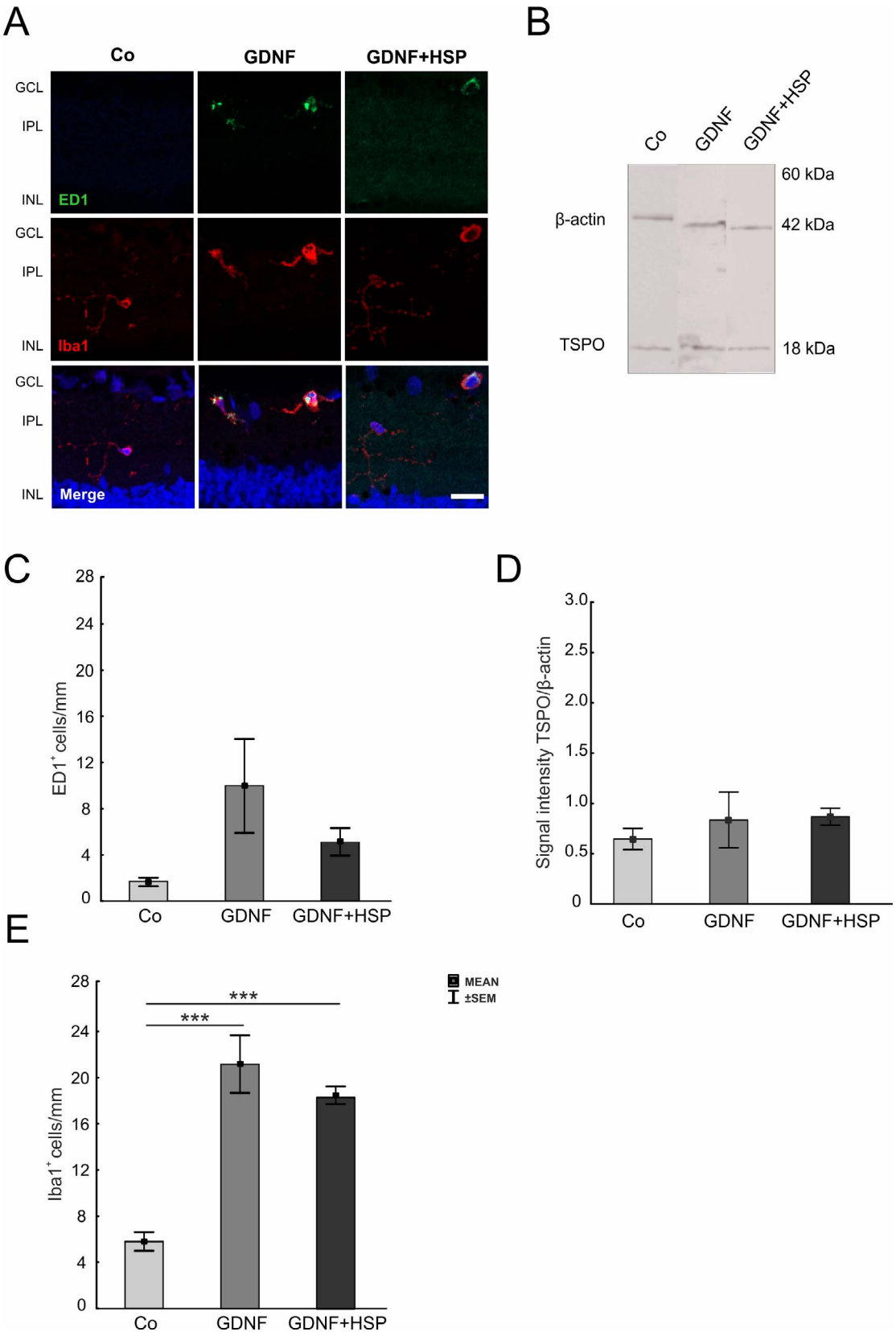

Figure 5. (**A**) Active microglia were stained with ED1 (*green*) and the whole population of microglial cells with Iba1 (*red*). Merged pictures show colocalization of both cells, which definitely identifies an active microglial cell. (**B**) Western blot pictures of TSPO and β-actin staining show exemplary bands of control and immunized animals. (**C**) No differences were observed between ED1 staining of the immunization groups and that of control (GDNF: $P = 0.07$; GDNF+HSP: $P = 0.7$). (**D**) In accordance to immunohistochemistry, Western blot analysis did not show any changes in TSPO signal intensity (GDNF: $P = 0.7$; GDNF+HSP: $P = 0.7$). (**E**) Iba1$^+$ cells were significantly increased in both of the immunization groups ($P < 0.001$). Values are mean ± SEM. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; IF, $n = 4$ to 5/group; Western blot (WB), $n = 3$ to 4/group. *Scale bar*: 20 μm.

Investigative Ophthalmology & Visual Science

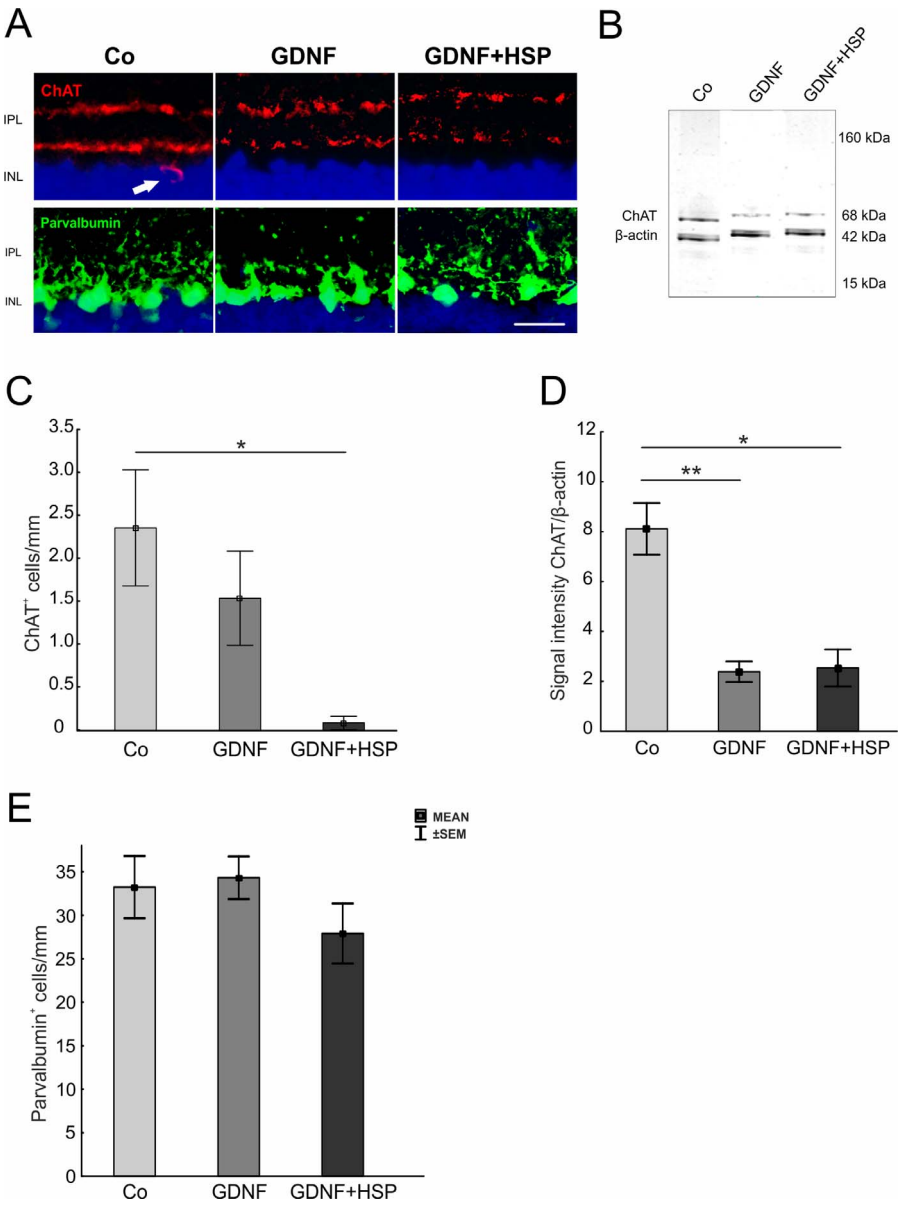

Figure 6. (**A**) Staining of cholinergic amacrine cells with ChAT (*red*) and staining of AII amacrine cells with parvalbumin (*green*). Cell nuclei were labeled with DAPI (*blue*). A ChAT^{+}-stained cell body is shown in the control group (*arrow*). (**B**) Exemplary Western blot images of ChAT and β-actin. (**C**) ChAT-stained cell bodies were counted. No differences were observed in the GDNF group ($P = 0.5$), but a significant loss was detected in the GDNF+HSP group ($P = 0.03$). (**D**) A significant decrease in ChAT signal intensity was evaluated by Western blot analysis in the GDNF ($P = 0.001$) and GDNF+HSP groups ($P = 0.002$). (**E**) No changes in AII amacrine cells were revealed with parvalbumin staining (GDNF: $P = 0.96$; GDNF+HSP: $P = 0.44$). Values are mean ± SEM. IPL, inner plexiform layer; INL, inner nuclear layer; IF, $n = 4$ to 5/group; Western blot (WB), $n = 3$ to 4/group. *Scale bar*: 20 µm.

Cell Loss of Particular Amacrine Cells

The cholinergic amacrine cells were labeled with a ChAT antibody, whereas the AII amacrine cells were marked with a parvalbumin antibody (Fig. 6A). Retinas of GDNF rats showed constant numbers of cholinergic amacrine cells (1.53 ± 0.55 cells/mm; $P = 0.5$) compared to controls (2.35 ± 0.68 cells/mm) (Fig. 6C). However, counting of the cell bodies in the GDNF+HSP group revealed a significant loss of cholinergic amacrine cells (0.08 ± 0.08 cells/mm; $P = 0.03$) (Fig. 6C).

We performed additional Western blot analysis with ChAT staining and measurement of bands at 68 kDa (Fig. 6B). Signal intensity was significantly decreased in both immunization groups (GDNF: 2.39 ± 0.41 units; $P = 0.001$; GDNF+HSP: 2.54 ± 0.74 units; $P = 0.002$) compared to controls (8.11 ± 1.04 units) (Fig. 6D).

AII amacrine cells were not affected in the GDNF group (Co: 33.24 ± 3.58 cells/mm; GDNF: 34.31 ± 2.45 cells/mm; $P = 0.9$) and in the GDNF+HSP group (27.91 ± 3.45 cells/mm; $P = 0.4$) (Fig. 6E).

In summary, no difference in AII amacrine cells was observed but a loss of cholinergic amacrine cells was noted in both immunization groups.

No Effects on Bipolar Cells

In order to identify different bipolar cells, retinal cross-sections were stained with PKCα and recoverin. Rod bipolar cells

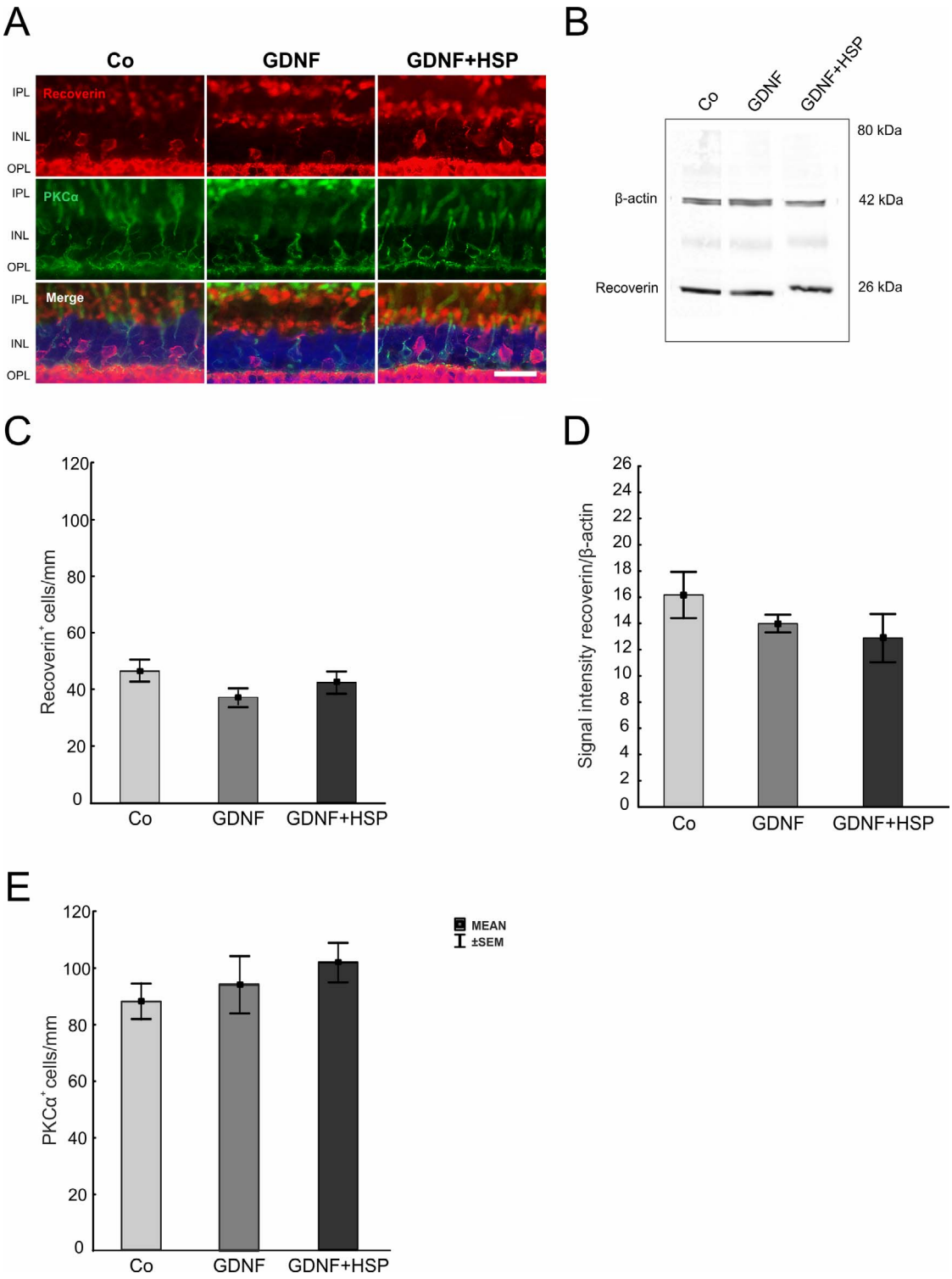

Figure 7. (**A**) Cone bipolar cells were evaluated using recoverin staining (*red*) and rod bipolar cells by using PKCα staining (*green*). (**B**) Representative pictures show Western blot lanes stained with recoverin and β-actin. (**C**) Recoverin$^+$ cells were not altered in either of the immunization groups compared to control (GDNF: $P = 0.1$; GDNF+HSP: $P = 0.6$). (**D**) Recoverin-stained Western blots did not detect differences between the groups (GDNF: $P = 0.5$; GDNF+HSP: $P = 0.3$). (**E**) Evaluation of rod bipolar cells with PKCα staining could detect no differences between immunization groups and control (GDNF: $P = 0.8$; GDNF+HSP: $P = 0.4$). Values are mean ± SEM. IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; IF, $n = 4$ to 5/group; Western blot (WB), $n = 3$ to 4/group. *Scale bar*: 20 μm.

(PKCα [green]) and cone bipolar cells (recoverin [red]) are located in the inner nuclear layer (Fig. 7A).

Staining with recoverin revealed no alterations in the GDNF group (Co: 46.67 ± 3.67 cells/mm; GDNF: 37.38 ± 2.67 cells/mm; $P = 0.1$) and in the GDNF+HSP group (42.49 ± 3.62 cells/mm; $P = 0.6$) (Fig. 7C). Western blot analysis was performed for recoverin staining with bands at 26 kDa and could detect no changes between immunization groups

(GDNF: 13.99 ± 0.67 units; $P = 0.5$; GDNF+HSP: 12.88 ± 1.84 units; $P = 0.3$) and controls (16.17 ± 1.76 units) (Fig. 7D). In regard to PKCα, no changes were noted in the GDNF group (94.05 ± 9.61 cells/mm; $P = 0.08$) compared to controls (87.83 ± 6.37 cells/mm) (Fig. 7E). Also, no differences were observed in GDNF+HSP animals (101.73 ± 7.07 cells/mm; $P = 0.4$).

No Alteration of Photoreceptors

Rhodopsin staining was used to evaluate changes in rods (Fig. 8A). The size of the stained area was measured. No differences could be detected between the control and the GDNF group (Co: 6.90 ± 0.47 area [%]/section; GDNF: 5.77 ± 1.07 area [%]/section; $P = 0.6$). Also in the GDNF+HSP group, rods were not altered compared to those of controls (GDNF+HSP: 6.91 ± 1.48 area [%]/section; $P = 0.99$) (Fig. 8C). Rhodopsin was also evaluated by Western blot analysis with a band at 39 kDa (Fig. 8B). We could detect a constant signal intensity in the GDNF group (239.12 ± 39.93 units; $P = 0.9$) and in the GDNF+HSP group (201.07 ± 38.3 units; $P = 0.9$) compared to controls (223.15 ± 28.52 units) (Fig. 8D).

Mostly L-cones are located in the rat retina.[27] Therefore, we labeled this cell type with opsin (Fig. 8A). Opsin$^+$ cells were counted, and the number was comparable in all groups (Co: 42.38 ± 2.81 cells/mm; GDNF: 39.35 ± 4.95 cells/mm; $P = 0.8$; GDNF+HSP: 37.58 ± 5.14 cells/mm; $P = 0.7$) (Fig. 8E). Therefore, we asserted that photoreceptors were not affected by the immunization.

Discussion

The involvement of the immune system in the disease of glaucoma was previously evaluated in different studies. Antibodies against ocular antigens can be detected in glaucoma patients, but it is still unclear if these antibodies are the cause or consequence of this disease.[7,8] In an autoimmune glaucoma animal model, glaucoma-related retinal changes could be demonstrated after immunization with ocular antigens.[15] In accordance with those studies, we now could show that immunization with two purified antigens led to neuronal cell loss in the retina. To our knowledge, this is the first study to demonstrate a loss of RGCs and other changes in the rat retina after immunization with GDNF and GDNF+HSP.

Heat shock proteins are small proteins which act intracellularly as chaperones. They are upregulated in situations of stress (e.g., glaucoma) and have cytoprotective effects. Tezel et al.[5] noted that autoantibodies to HSP27 were increased most prominently in glaucoma patients without an elevated IOP and that HSP27 was identified as one of the important antigens in normal tension glaucoma patients.[10] It was further demonstrated that exogenously applied HSP27 antibody binds to actin filaments and can lead to a cellular breakdown.[28] In previous studies, immunization with HSP27 alone resulted in loss of RGCs[15,17] and was hypothesized to act as an antigenic stimulus after immunization.[15]

To find out more about specific antigens that might contribute to glaucomatous damage, we examined possible effects of another antigen, which might be of interest, namely GNDF. GDNF is a distant member of the TGF-β superfamily and a potent survival factor for dopamine neurons. It acts neuroprotectively in animal models of Parkinson disease, which led to the assumption it could be a useful drug for the treatment of neurodegenerative diseases.[29] In vitro studies have recently demonstrated a neuroprotective effect of GDNF on the porcine retina.[30] In contrast, in a pilot study, GDNF led to loss of RGCs after immunization and was therefore also chosen for this study. Here, we also combined GDNF with HSP27 to reveal whether this combination led to additional cell loss or not.

Retinal Ganglion Cells Loss

Immunization with GDNF led to loss of RGCs, although it is known to be neuroprotective. We also proposed an antigenic reaction to GDNF immunization, as there is no neuroprotection but actually a damage of neuronal cells after immunization. Interestingly, immunization with the combination of GDNF and HSP27 did not lead to a greater loss of RGCs. One would suspect an additive loss of neuronal cells after the immunization with the combination of GDNF and HSP27, as immunization with HSP27 alone leads to RGC loss.[15,17] In another study conducted by our group, we found a comparable result after immunization with a combination of HSP27 and the S100B protein.[16] Here, immunization with the combination did not lead to a greater loss of RGCs than the immunization with S100B alone. The reason for these results could be explained by the duration of the study after immunization. The cell loss in a study with HSP27 alone did not occur before five weeks after immunization.[17] Possibly, the cell loss observed in the GDNF+HSP group was triggered only by GDNF 4 weeks after immunization.

Glia Cells

Glial cells are an important factor for neuronal health. Astrocytes and Müller cells are macroglial cells and control homeostasis. They eliminate neuronal waste, such as carbon dioxide and ammonia, and protect neurons by recycling neurotransmitters in healthy tissues,[31] but there are still discussions about whether gliosis acts as a neuroprotectant or a neurodestructant during neurodegeneration.[32] Müller cells form radial columns throughout the retina, whereas astrocytes are mostly located in the nerve fiber layer.[31] We evaluated astrocytes via GFAP and Müller cells via vimentin staining. Previous studies showed an increased expression of GFAP after neuronal damage, as seen in the disease of glaucoma.[32] Only a tendency of this effect could be noted in our study in the GDNF group. No gliosis was noted in the GDNF+HSP group. In a study by Wax et al.,[15] an increase of GFAP expression was observed after immunization with HSP27 alone. In a previous study by our group, using S100B and HSP27, we also noted gliosis in the combination group.[16] Therefore, one could have presumed a stronger reaction of astrocytes. On the contrary, a study of nerve regeneration after spinal cord injury showed a decreased production of GFAP and reduced hypertrophy of astrocytes after GDNF treatment in combination with Schwann cells.[33] Although GDNF alone led to a tendency of increase in GFAP staining, no increase was seen when it was combined with HSP27. Therefore, we presume that there are interactions between GDNF and HSP27.

Müller cells are upregulated in situations of retinal disease like retinal detachment or high-pressure glaucoma.[34,35] They can be detected by vimentin staining,[36] but in situations of neurodegeneration, the expression of GFAP is also increased in these cells. We could not detect any differences in regard to vimentin. The results are similar to previous results of our group, where we observed only increased GFAP expression and no vimentin increase after immunization with S100B and HSP27.[16]

Microglia are the resident macrophages of the retina. These cells show immune activity, and they are important for the development and maintenance of the neuronal network

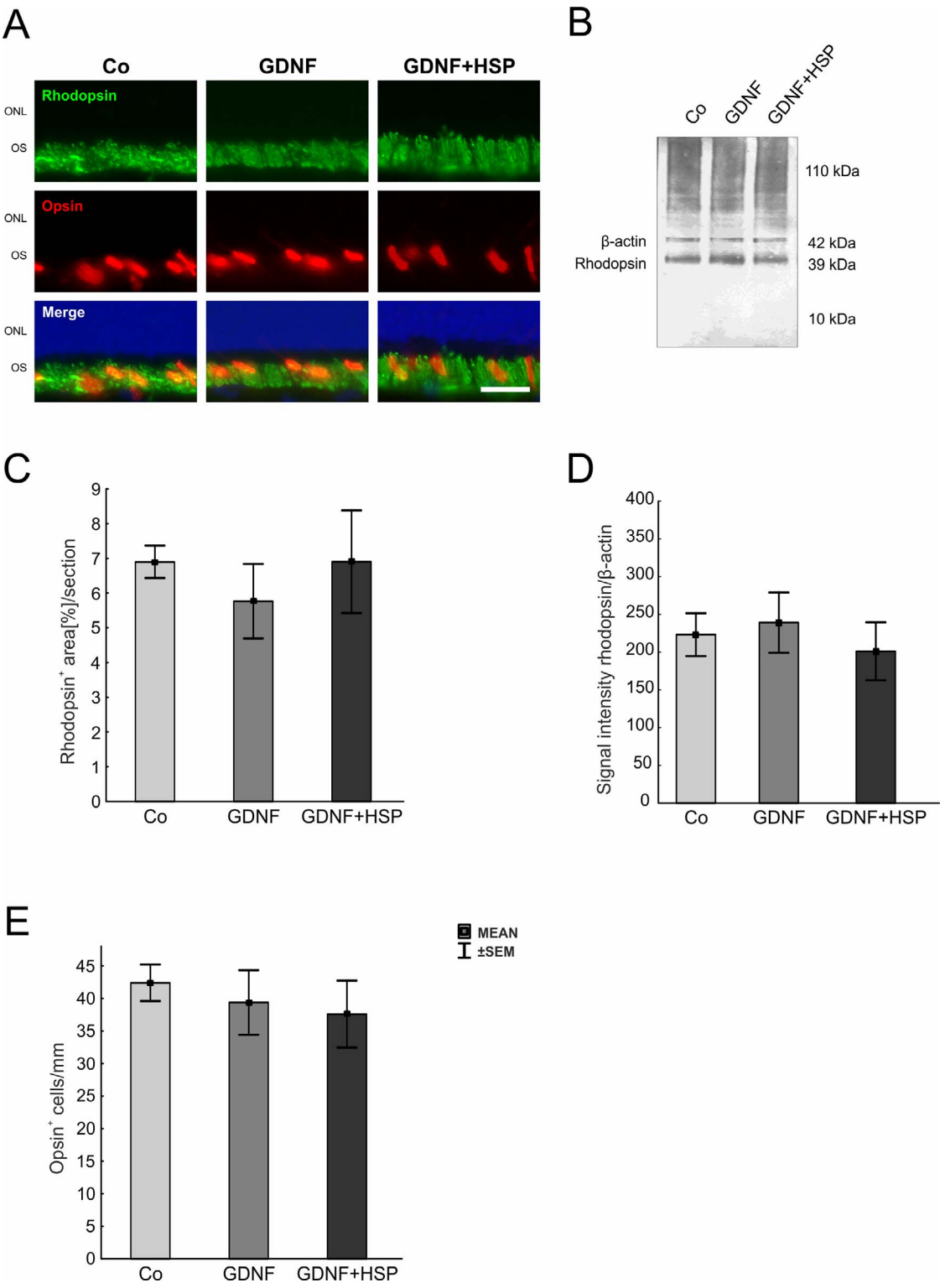

Figure 8. (**A**) Rhodopsin (rods, *green*) and opsin (L-cones, *red*) staining of retinal sections was performed to evaluate photoreceptors. (**B**) Exemplary Western blot images after rhodopsin and β-actin staining. (**C**) No changes were detected after rhodopsin staining for immunohistochemistry (GDNF: $P = 0.6$; GDNF+HSP: $P = 0.99$). (**D**) Western blot results of rhodopsin could reveal no differences between immunization groups and control (GDNF: $P = 0.9$; GDNF+HSP: 0.9). (**E**) No differences could be seen with regard to opsin staining in the control group (GDNF: $P = 0.8$; GDNF+HSP: $P = 0.7$). Values mean ± SEM. ONL, outer nuclear layer; OS, outer segment; IF, $n = 4$ to 5/group; WB, $n = 3$ to 4/group. *Scale bar*: 20 μm.

and for tissue homeostasis.[37] After retinal injury, they can be stimulated to undertake phagocytosis of degenerating retinal neurons.[31] They can be in a resting (inactive) or active state.[37] An increase in microglia was detected in different models of glaucoma, for example, after induced hypertension[38] or optic nerve damage.[39] Also, an increase in activated microglia could be evaluated in the autoimmune glaucoma model 14 days after immunization with optic nerve antigen

and S100.[20] In accordance with this, we could detect a significant increase of Iba1$^+$ microglia. However, activated microglia showed only a tendency of increase in the GDNF group. Strong microglial reactions have been found in early stages of retinal damage[20] and decreased between 3 and 12 weeks.[39] Our results after 4 weeks will therefore likely represent the decreasing phase.

Decline of Amacrine Cells

Amacrine cells are interneurons at the second synaptic level, yet there are currently no data for whether immunization with GDNF or HSP27 affects this cell type. Amacrine cells are a highly diverse class of retinal neurons. Those that use glycine as a neurotransmitter are small-field amacrine cells. The most common type of small-field amacrine cells in the mammalian retina is the AII amacrine cell. It connects rod and cone photoreceptor pathways.[40,41] The cholinergic amacrine cells, on the other hand, are known to use acetylcholine (ACh) as the neurotransmitter. They can be differentiated into two types. One is the ACh-a type, whose cell bodies are located in the inner nuclear layer. The cell bodies of the second type, called ACh-b, are displaced to the ganglion cell layer.[21,42] Here, we focused on the ACh-a type by labeling them with an anti-ChAT antibody. A loss of cholinergic amacrine cells was found only in the GDNF+HSP group by immunohistochemistry but in both immunization groups by Western blot analysis. Differences between results can be explained with the use of proteins of the whole retina for a Western blot and the evaluation of particular retinal layers in immunohistochemistry. Cholinergic amacrine cells are sending their long dendrites into the inner plexiform layer, seen as stratification. Loss of stratification is not included in the statistics when the cell bodies are counted. Therefore, the Western blot analysis seems to be more sensible with this cell type. The environment in surrounding tissues will change after a loss of RGCs, and cholinergic amacrine cells can be affected, as was demonstrated in a mouse model of optic nerve injury.[43] Based on our results in regard to the missing macroglia gliosis, we presume that the cholinergic amacrine cells were more vulnerable and therefore a destructive effect of GDNF immunization is apparent at this point in time.

The AII amacrine cells were not affected through the immunization. As already mentioned, AII amacrine cells are connected with many dopamine amacrine cells.[44] Since GDNF is known to act neuroprotectively on dopamine neurons,[29] there could be a positive effect on the neighboring AII amacrine cells.

No Effect on Bipolar Cells

Cell bodies of bipolar cells are also located in the inner nuclear layer and are sending their dendrites into the outer plexiform layer. They are connected either with rods or cones.[45] We used PKCα to label the rod bipolar cells and recoverin for cone bipolar cells.[46] Neither of these cell types was affected at 4 weeks after immunization. In an ocular hypertension model, a loss of dendritic processes of rod bipolar cells was documented after 3 weeks.[47] In another study, a significant reduction of bipolar cells was not detected before 5 weeks after elevation of IOP.[36] A study by Park et al.[48] revealed a decrease in synapses after loss of RGCs due to ocular hypertension and it would be of interest if the same effect occurs IOP-independently after ganglion cells loss due to immunization at later points in time.

No Damage of Photoreceptors

Cells of the outer retina, the photoreceptors, remained unaffected after immunization. In different animal models of hypertension glaucoma, a loss of photoreceptors was noted.[49,50] Until now, there have not been many studies of photoreceptors in normal tension glaucoma models, but the retinal layers were well organized in a previous study of the autoimmune glaucoma model.[16] Therefore, we would not have presumed changes in photoreceptors in this model at this stage of degeneration. These results indicate various mechanisms in glaucoma pathology. It is important to evaluate high-pressure as well as normal tension glaucoma in adequate animal models.

Conclusions

In summary, our findings demonstrated for the first time that immunizing rats with GDNF alone or in combination with HSP27 led to a significant loss of RGCs and an increase in inactive microglia after 4 weeks. Additionally, both groups showed a loss of cholinergic amacrine cells. In the GDNF and the GDNF+HSP group we detected only a slight or no gliosis. Activation of astrocytes, which was seen after immunization with HSP alone, seems to be inhibited by GDNF. This is the reason why we presume complex processes between GDNF and HSP27. The immunization with GDNF is the cause for retinal damage in our study.

It will be the task of following studies to evaluate the retina at a later point in time after immunization. Furthermore, varying concentrations of antigens could reveal concentration dependent neuroprotective or neurodestructive mechanisms. This way a further understanding of the effects of immunization with GDNF and GDNF+HSP and the interaction between the two antigens can be reached. The aim of many current glaucoma model studies is to find a method for neuroprotection. Hence, it is very important to clarify if a substance or a combination acts as a neuroprotective or neurodestructive agent.

Acknowledgments

This work was supported by German Research Foundation (DFG) Grant JO 886/1-3. The authors alone are responsible for the content and writing of the paper.

Disclosure: **C. Casola**, None; **S. Reinehr**, None; **S. Kuehn**, None; **G. Stute**, None; **B.M. Spiess**, None; **H.B. Dick**, None; **S.C. Joachim**, None

References

1. Tezel G, Wax MB. Glaucoma. *Chem Immunol Allergy*. 2007; 92:221–227.
2. Sommer A, Tielsch JM, Katz J, et al. Relationship between intraocular pressure and primary open angle glaucoma among white and black Americans. The Baltimore Eye Survey. *Arch Ophthalmol*. 1991;109:1090–1095.
3. Tezel G, Wax MB. The immune system and glaucoma. *Curr Opin Ophthalmol*. 2004;15:80–84.
4. Wax MB, Tezel G, Saito I, et al. Anti-Ro/SS-A positivity and heat shock protein antibodies in patients with normal-pressure glaucoma. *Am J Ophthalmol*. 1998;125:145–157.
5. Tezel G, Seigel GM, Wax MB. Autoantibodies to small heat shock proteins in glaucoma. *Invest Ophthalmol Vis Sci*. 1998; 39:2277–2287.
6. Joachim SC, Pfeiffer N, Grus FH. Autoantibodies in patients with glaucoma: a comparison of IgG serum antibodies against

retinal, optic nerve, and optic nerve head antigens. *Graefes Arch Clin Exp Ophthalmol*. 2005;243:817–823.

7. Grus FH, Joachim SC, Hoffmann EM, Pfeiffer N. Complex autoantibody repertoires in patients with glaucoma. *Mol Vis*. 2004;10:132–137.
8. Joachim SC, Wuenschig D, Pfeiffer N, Grus FH. IgG antibody patterns in aqueous humor of patients with primary open angle glaucoma and pseudoexfoliation glaucoma. *Mol Vis*. 2007;13:1573–1579.
9. Tezel G, Yang J, Wax MB. Heat shock proteins, immunity and glaucoma. *Brain Res Bull*. 2004;62:473–480.
10. Wax MB, Tezel G, Kawase K, Kitazawa Y. Serum autoantibodies to heat shock proteins in glaucoma patients from Japan and the United States. *Ophthalmology*. 2001;108:296–302.
11. Fatemi I, Shamsizadeh A, Roohbakhsh A, Ayoobi F, Sanati MH, Motevalian M. Increase in mRNA level of Orexin1 and 2 receptors following induction of experimental autoimmune encephalomyelitis in mice. *Iran J Allergy Asthma Immunol*. 2016;15:20–26.
12. Martin BN, Wang C, Zhang CJ, et al. T cell-intrinsic ASC critically promotes T17-mediated experimental autoimmune encephalomyelitis. *Nat Immunol*. 2016;17:583–592.
13. Laspas P, Gramlich OW, Muller HD, et al. Autoreactive antibodies and loss of retinal ganglion cells in rats induced by immunization with ocular antigens. *Invest Ophthalmol Vis Sci*. 2011;52:8835–8848.
14. Joachim SC, Gramlich OW, Laspas P, et al. Retinal ganglion cell loss is accompanied by antibody depositions and increased levels of microglia after immunization with retinal antigens. *PLoS One*. 2012;7:e40616.
15. Wax MB, Tezel G, Yang J, et al. Induced autoimmunity to heat shock proteins elicits glaucomatous loss of retinal ganglion cell neurons via activated T-cell-derived fas-ligand. *J Neurosci*. 2008;28:12085–12096.
16. Casola C, Schiwek JE, Reinehr S, et al. S100 alone has the same destructive effect on retinal ganglion cells as in combination with HSP 27 in an autoimmune glaucoma model. *J Mol Neurosci*. 2015;56:228–236.
17. Joachim SC, Grus FH, Kraft D, et al. Complex antibody profile changes in an experimental autoimmune glaucoma animal model. *Invest Ophthalmol Vis Sci*. 2009;50:4734–4742.
18. Cuny CS, Joachim SC, Gramlich OW, Gottschling PF, Pfeiffer N, Grus FH. Repeated intraocular pressure measurement in awake Lewis rats does not bias retinal ganglion cell survival. *Curr Eye Res*. 2010;35:1034–1039.
19. Horstmann L, Schmid H, Heinen AP, Kurschus FC, Dick B, Joachim SC. Inflammatory demyelination induces glia alterations and ganglion cell loss in the retina of an experimental autoimmune encephalomyelitis model. *J Neuroinflammation*. 2013;10:120.
20. Noristani R, Kuehn S, Stute G, et al. Retinal and optic nerve damage is associated with early glial responses in an experimental autoimmune glaucoma model. *J Mol Neurosci*. 2016;58:470–482.
21. Rodriguez AR, de Sevilla Muller LP, Brecha NC. The RNA binding protein RBPMS is a selective marker of ganglion cells in the mammalian retina. *J Comp Neurol*. 2014;522:1411–1443.
22. Jiang SM, Zeng LP, Zeng JH, Tang L, Chen XM, Wei X. beta-III-Tubulin: a reliable marker for retinal ganglion cell labeling in experimental models of glaucoma. *Int J Ophthalmol*. 2015;8:643–652.
23. Ching AS, Kuhnast B, Damont A, Roeda D, Tavitian B, Dolle F. Current paradigm of the 18-kDa translocator protein (TSPO) as a molecular target for PET imaging in neuroinflammation and neurodegenerative diseases. *Insights Imaging*. 2012;3:111–119.
24. Karlstetter M, Nothdurfter C, Aslanidis A, et al. Translocator protein (18 kDa) (TSPO) is expressed in reactive retinal microglia and modulates microglial inflammation and phagocytosis. *J Neuroinflammation*. 2014;11:3.
25. Chen MK, Guilarte TR. Translocator protein 18 kDa (TSPO): molecular sensor of brain injury and repair. *Pharmacol Ther*. 2008;118:1–17.
26. Wang M, Wang X, Zhao L, et al. Macroglia-microglia interactions via TSPO signaling regulates microglial activation in the mouse retina. *J Neurosci*. 2014;34:3793–3806.
27. Ortin-Martinez A, Jimenez-Lopez M, Nadal-Nicolas FM, et al. Automated quantification and topographical distribution of the whole population of S- and L-cones in adult albino and pigmented rats. *Invest Ophthalmol Vis Sci*. 2010;51:3171–3183.
28. Tezel G, Wax MB. The mechanisms of hsp27 antibody-mediated apoptosis in retinal neuronal cells. *J Neurosci*. 2000;20:3552–3562.
29. Saarma M. GDNF - a stranger in the TGF-beta superfamily? *Eur J Biochem*. 2000;267:6968–6971.
30. Taylor L, Arner K, Engelsberg K, Ghosh F. Effects of glial cell line-derived neurotrophic factor on the cultured adult full-thickness porcine retina. *Curr Eye Res*. 2013;38:503–515.
31. Goldman D. Müller glial cell reprogramming and retina regeneration. *Nat Rev Neurosci*. 2014;15:431–442.
32. Tezel G. The role of glia, mitochondria, and the immune system in glaucoma. *Invest Ophthalmol Vis Sci*. 2009;50:1001–1012.
33. Deng LX, Hu J, Liu N, et al. GDNF modifies reactive astrogliosis allowing robust axonal regeneration through Schwann cell-seeded guidance channels after spinal cord injury. *Exp Neurol*. 2011;229:238–250.
34. Wang X, Tay SS, Ng YK. An immunohistochemical study of neuronal and glial cell reactions in retinae of rats with experimental glaucoma. *Exp Brain Res*. 2000;132:476–484.
35. Verardo MR, Lewis GP, Takeda M, et al. Abnormal reactivity of Müller cells after retinal detachment in mice deficient in GFAP and vimentin. *Invest Ophthalmol Vis Sci*. 2008;49:3659–3665.
36. Hernandez M, Rodriguez FD, Sharma SC, Vecino E. Immunohistochemical changes in rat retinas at various time periods of elevated intraocular pressure. *Mol Vis*. 2009;15:2696–2709.
37. Vecino E, Rodriguez FD, Ruzafa N, Pereiro X, Sharma SC. Glia-neuron interactions in the mammalian retina. *Prog Retin Eye Res*. 2016;51:1–40.
38. Trost A, Motloch K, Bruckner D, et al. Time-dependent retinal ganglion cell loss, microglial activation and blood-retina-barrier tightness in an acute model of ocular hypertension. *Exp Eye Res*. 2015;136:59–71.
39. Cen LP, Han M, Zhou L, et al. Bilateral retinal microglial response to unilateral optic nerve transection in rats. *Neuroscience*. 2015;311:56–66.
40. Demb JB, Singer JH. Intrinsic properties and functional circuitry of the AII amacrine cell. *Vis Neurosci*. 2012;29:51–60.
41. Kolb H, Famiglietti EV. Rod and cone pathways in the inner plexiform layer of cat retina. *Science*. 1974;186:47–49.
42. Vaney DI. "Coronate" amacrine cells in the rabbit retina have the "starburst" dendritic morphology. *Proc R Soc Lond B Biol Sci*. 1984;220:501–508.
43. Bernstein SL, Guo Y. Changes in cholinergic amacrine cells after rodent anterior ischemic optic neuropathy (rAION). *Invest Ophthalmol Vis Sci*. 2011;52:904–910.
44. Kolb H, Cuenca N, Dekorver L. Postembedding immunocytochemistry for GABA and glycine reveals the synaptic relationships of the dopaminergic amacrine cell of the cat retina. *J Comp Neurol*. 1991;310:267–284.

Investigative Ophthalmology & Visual Science

45. Kolb H. Organization of the outer plexiform layer of the primate retina: electron microscopy of Golgi-impregnated cells. *Philos Trans R Soc Lond B Biol Sci*. 1970;258:261–283.
46. Milam AH, Dacey DM, Dizhoor AM. Recoverin immunoreactivity in mammalian cone bipolar cells. *Vis Neurosci*. 1993;10:1–12.
47. Cuenca N, Pinilla I, Fernandez-Sanchez L, et al. Changes in the inner and outer retinal layers after acute increase of the intraocular pressure in adult albino Swiss mice. *Exp Eye Res*. 2010;91:273–285.
48. Park HY, Kim JH, Park CK. Alterations of the synapse of the inner retinal layers after chronic intraocular pressure elevation in glaucoma animal model. *Mol Brain*. 2014;7:53.
49. Fernandez-Sanchez L, de Sevilla Muller LP, Brecha NC, Cuenca N. Loss of outer retinal neurons and circuitry alterations in the DBA/2J mouse. *Invest Ophthalmol Vis Sci*. 2014;55:6059–6072.
50. Ortin-Martinez A, Salinas-Navarro M, Nadal-Nicolas FM, et al. Laser-induced ocular hypertension in adult rats does not affect non-RGC neurons in the ganglion cell layer but results in protracted severe loss of cone-photoreceptors. *Exp Eye Res*. 2015;132:17–33.

Investigative Ophthalmology & Visual Science